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Journal of Chromatography A, 786 (1997) 57–65

JOURNAL OF CHROMATOGRAPHY A

Determination of γ-radiation induced products in aqueous solutions of tryptophan and synthesis of 4-, 6- and 7-hydroxytryptophan

Barbara van Wickern, Burkhardt Müller, Thomas Simat*, Hans Steinhart

Institute of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, D-20146 Hamburg, Germany

Received 6 March 1997; received in revised form 1 May 1997; accepted 1 May 1997

Abstract

A HPLC method with UV and fluorescence detection was developed to determine γ-radiation induced products in aqueous solutions of tryptophan. Therefore 4-, 6- and 7-hydroxytryptophan were synthesized in purities which allow their use as reference substances for analytical studies. Oxindolylalanine, N-formylkynurenine, 4-, 5, 6- and 7-hydroxytryptophan were determined as the main radiation induced products. © 1997 Elsevier Science B.V.

Keywords: γ-Radiation; Tryptophan; Hydroxytryptophan

1. Introduction

The preservation of food by treatment with ionizing radiation has been established in several countries. In the last few years various methods for the detection of irradiated food have been developed, but it has so far proved difficult to detect radiation induced products in food with a high protein content.

Tryptophan (TRP) is amongst the most reactive amino acids and due to its importance as an essential amino acid, the effect of irradiation on TRP has been investigated intensively by many working groups [1–5].

During the γ-radiation of water or of aqueous solutions following reactive primary radiation products are formed:

$$H_2O \rightarrow H\cdot + e^-_{aq} + OH\cdot \quad (1)$$

The OH· radical is a strong oxidizing agent and its major reaction with TRP is addition to the benzene- or to the pyrrole-ring [6,7]. The reducing transients e^-_{aq} and H· are converted into peroxy radicals in the presence of oxygen. The major product of their reaction with TRP is N-formylkynurenine (NFK) [7].

In irradiated aqueous solutions of TRP, NFK [1,2,4,5], kynurenine (KYN) [2,4,5], oxindolylalanine (OIA) [1,2] and hydroxytryptophan isomers (OH-TRP) [1,2,5] have been identified as the main radiation induced products by various authors. Because of the lack of reference substances, or because the authors had no suitable methods for separation of all radiation products, there are no reports on the quantitative determination of these products.

The aim of this study was to develop a high-performance liquid chromatograpy (HPLC) method with UV- and fluorescence (FL) detection which would allow the determination of stable radiation induced products of TRP. Only UV-active substances with an intact α-amino acid structure have been taken into consideration. In addition, a fast method to

*Corresponding author.

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PII S0021-9673(97)00516-5

synthesize the aphysiological OH-TRP isomers 4-, 6- and 7-OH-TRP to a purity that allows their use as reference substances was developed. The study examined, wether one radiation induced product of TRP or a pattern of products could be used as marker for irradiated food with a high protein content.

2. Material and methods

2.1. Chemicals

7-Benzyloxytryptophan was obtained from Aldrich (Steinheim, Germany). Dowex-50W (cross-linkage 2%, 200–400 mesh, H^+ form) cation-exchange resin and Dowex-1 (cross-linkage 2%, 200–400 mesh, CH_3COO^- form) anion-exchange resin were from Sigma (Deisenhofen, Germany). All other chemicals for synthesis were from Merck (Darmstadt, Germany), Aldrich or Fluka (Neu-Ulm, Germany).

D,L-Kynurenine (KYN) Sigma and 5-hydroxytryptophan (5-OH-TRP) Merck were purchased in analytical quality. L-Tryptophan (TRP) was provided by Degussa (Hanau, Germany). Oxindolylalanine (OIA), dioxindolylalanine (DIOIA) and N-formylkynurenine (NFK) were synthesized as described previously [8].

Methanol (MeOH) (gradient-grade), acetonitrile (MeCN) (gradient-grade) and trifluoroacetic acid (TFA) (Uvasol) were purchased from Merck.

Bidistilled water was produced with a Hereaeus-Destamat Bi18E bidistillator.

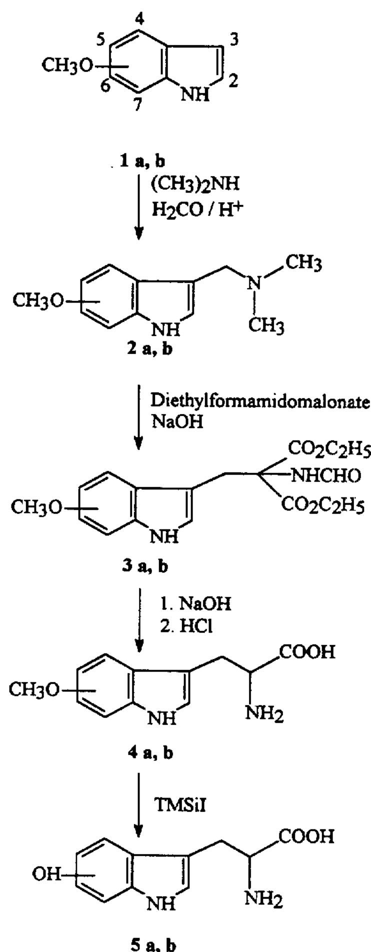


Fig. 1. Synthesis scheme of 4- and 6-OH-TRP.

2.2. Synthesis of 4-, 6- and 7-hydroxytryptophan

2.2.1. 4- and 6-Methoxyindole (Fig. 1, 1a,b)

4- and 6-Methoxyindole were synthesized via electrophilic substitution of 3-methoxyaniline with chloroacetonitrile in the presence of boron trichloride and titanium tetrachloride followed by cyclic reduction according to Sugasawa et al. [9].

2.2.2. 4-Methoxygramine (2a)

A solution of 1.0 g (6.8 mmol) 4-methoxyindole in 7 ml dioxane was added dropwise to an ice-cooled solution of 7 ml dioxane, 7 ml glacial acetic acid, 562 µl (5.3 mmol) of 37% aqueous formaldehyde and 947 µl (7.5 mmol) of 40% aqueous dimethylamine. The clear solution was stirred and cooled for 2 h, then allowed to warm up and stirred for another 1.5 h. Afterwards, first 50 ml water and then 20 ml of 2 *M* NaOH were added to the mixture. The product precipitated as a white solid. The gramine was filtered off, washed with water and dried, yielding 1.0 g (4.9 mmol) 72% of 4-methoxygramine.

Melting point: 88°C.

^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.27$ (s, 6H, 2 CH_3), 3.80 (s, 2H, CH_2), 3.86 (s, 3H, CH_3O), 6.45 (d, 1H, H-7), 6.92 (d, 1H, H-5), 6.93 (s, 1H, H-2), 7.03 (dd, 1H, H-6), 8.37 (bs, 1H, N-H) ppm. $^3J_{5,6}=7.8$, $^3J_{6,7}=7.6$ Hz.

^{13}C NMR (100 MHz, $CDCl_3$): $\delta=44.6$ (CH_3),

54.6 (CH_2), 54.6 (CH_3O), 99.3 (C-7), 104.0 (C-2), 112.9 (C-3), 117.2 (C-3a), 123.0 (C-6), 122.1 (C-5), 137.4 (C-7a), 154.4 (C-4) ppm.

MS (70 eV, EI): m/z (%)=204 (18, M^+), 203 (5), 161 (22), 160 (100), 159 (62), 158 (4), 132 (8), 130 (52), 129 (44), 128 (9), 117 (20), 116 (8), 103 (12), 102 (23), 89 (15), 78 (3), 77 (9), 75 (7), 63 (23), 58 (12), 51 (7), 45 (13).

2.2.3. 6-Methoxygramine (2b)

A solution of 800 mg (5.4 mmol) 6-methoxyindole and 5.6 ml dioxane was added over the course of 20 min to an ice-cooled solution of 5.6 ml dioxane, 5.6 ml glacial acetic acid, 450 μl (6.0 mmol) of 37% aqueous formaldehyde and 760 μl (6.0 mmol) of 40% aqueous dimethylamine. The solution was stirred and cooled for 1.5 h. 20 ml water was added the mixture and alkalized with an excess of 1 *M* ammonia. The turbid mixture was extracted three times with 15 ml methylenechloride. The combined organic layers were dried and concentrated. The brownish residue was dissolved in 5 ml methylenechloride and treated with hexane until the crystallisation started. After standing overnight at −18°C, the gramine was filtered off and dried, yielding 1.09 g (5.3 mmol) 98% of 6-methoxygramine.

Melting point: 80–85°C.

^1H NMR (400 MHz, $CDCl_3$): δ=2.26 (s, 6H, 2 CH_3), 3.61 (s, 2H, CH_2), 3.83 (s, 3H, CH_3O), 6.79 (dd, 1H, H-5), 6.83 (d, 1H, H-7), 7.02 (s, 1H, H-2), 7.55 (d, 1H, H-4), (bs, 1H, N-H) ppm. $^3J_{4,5}$=8.8, $^4J_{5,7}$=1.9 Hz.

^{13}C NMR (100 MHz, $CDCl_3$): δ=45.0 (CH_3), 54.1 (CH_2), 55.2 (CH_3O), 94.3 (C-7), 108.9 (C-5), 113.1 (C-3), 119.5 (C-4), 122.8 (C-3a), 124.0 (C-2), 137.1 (C-7a), 156.0 (C-6) ppm.

MS (70 eV, EI): m/z (%)=204 (32, M^+), 203 (5), 188 (3), 161 (22), 160 (100), 159 (22), 145 (10), 144 (4), 129 (4), 117 (12), 102 (3), 90 (4), 89 (9), 77 (2), 63 (4), 58 (4), 57 (2).

2.2.4. Ethyl-β-(4-methoxyindolyl-3)-α-carbethoxy-α-formamidopropionate (3a)

A suspension of 200 mg (980 μmol) 4-methoxygramine, 250 mg (1.23 mmol) diethylformamidomalonate, 20 mg (490 μmol) powdered NaOH in 10 ml toluene was heated under reflux for 4 h, while a vigorous stream of nitrogen was bubbled through. The hot suspension was filtered and allowed to cool down. The clear filtrate was washed with water, 1 *M* HCl, 1 *M* NaOH and again with water and dried. The solution was treated with petroleum ether until turbid and allowed to stand overnight at room temperature. The product was filtered off, washed with petroleum ether and dried. 210 mg (560 μmol) 57% ester were obtained.

Melting point: 212–215°C.

^1H NMR (400 MHz, $CDCl_3$): δ=1.23 (t, 6H, CH_3-CH_2), 3.87 (s,3H, CH_3O), 3.92 (s, 2H, CH_2), 4.22 (q, 4H, CH_2-CH_3), 6.43 (d, 1H, H-7), 6.69 (bs, 1H, *NH*-CHO), 6.77 (s, 1H, H-2), 6.93 (d, 1H, H-5), 7.03 (dd, 1H, H-6), 8.06 (s, 1H, CHO), 8.11 (bs, 1H, NH-Indol), (s, 1H, CHO) ppm. $^3J_{5,6}$=8.0, $^3J_{6,7}$=7.6 Hz.

^{13}C NMR (100 MHz, $CDCl_3$): δ=13.5 (CH_3-CH_2), 29.1 (CH_2), 5.4 (CH_3O), 62.0 (CH_2-CH_3), 66.8 ($C_{quart.}$-aliphat.), 99.2 (C-7), 104.1 (C-2), 108.5 (C-2), 117.3 (C-3a), 122.0 (C-6), 122.4 (C-5), 131.1 (C-7a), 154.0 (C-4), 159.3 (CHO), 167.3 (CO_2-Et) ppm.

MS (70 eV, EI): m/z (%)=376 (5, M^+), 363 (10), 317 (8), 232 (3), 161 (11), 160 (100), 159 (12), 130 (28), 85 (7), 74 (5), 63 (4).

2.2.5. Ethyl-β-(6-methoxyindolyl-3)-α-carbethoxy-α-formamidopropionate (3b)

A mixture of 1.09 g (5.3 mmol) 6-methoxygramine, 1.36 g (6.7 mmol) diethylformamidomalonate and 120 mg (2.9 mmol) powdered NaOH and 70 ml toluene was heated under reflux for 22 h, while a vigorous stream of nitrogen was bubbled through. The hot suspension was filtered and allowed to cool down. The clear filtrate was washed with water, 1 *M* HCl, 1 *M* NaOH, and again with water and dried. The solvent was evaporated, yielding 1.42 g (3.9 mmol) 73% ester.

Melting point: 198–201°C.

^1H NMR (400 MHz, $CDCl_3$): δ=1.27 (t, 6H, CH_3-CH_2), 3.81 (s, 3H, CH_3O), 3.91 (s, 2H, CH_2), 4.22 (q, 4H, CH_2-CH_3), 6.72 (dd, 1H, H-5), 6.79 (s, 1H, H-2), 6.80 (d, 1H, H-7), 6.81 (bs, 1H, *NH*-CHO), 7.36 (d, 1H, H-4), 7.99 (bs, 1H, NH-Indol), (s, 1H, CHO) ppm. $^3J_{4,5}$=8.80, $^4J_{5,7}$=2.41 Hz.

^{13}C NMR (100 MHz, $CDCl_3$): δ=14.2 (CH_3-CH_2), 27.6 (CH_2), 55.3 (CH_3O), 62.8 (CH_2-CH_3), 66.7 ($C_{quart.}$-aliphat.), 94.8 (C-7), 107.7 (C-3), 109.0 (C-5), 119.1 (C-4), 122.3 (C-2), 122.6 (C-3a), 136.2 (C-7a), 156.9 (C-6), 160.0 (CHO), 167.2 (CO_2-Et) ppm.

MS (70 eV, EI): m/z (%)=376 (5, M^+), 363 (10), 317 (5), 188 (3), 161 (11), 160 (100), 145 (8), 117 (3), 85 (7), 74 (5), 57 (2).

2.2.6. 4- and 6-Methoxytryptophan (4a,b)

A mixture of 663 mg (17 mmol) NaOH, 500 mg (1.4 mmol) of the respective methoxyformamido-ester (3a,b) and 15 ml water was refluxed for 3 h. After neutralization with 2 *M* HCl, the amino malonic acid precipitated. After further addition of 700 µl (1.4 mmol) 2 *M* HCl refluxing was continued for 30 min and the solid went completely into solution with the evolution of CO_2. The hot solution was filtered and the pH of the yellow filtrate was adjusted to 6.8 (isoelectric point of tryptophan) with 1 *M* ammonia. During removal of the solvent the amino acid precipitated. The solid was collected, washed with cooled ethanol and dried, yielding 290 mg (1.3 mmol) 94% 4-methoxytryptophane and 294 mg (1.3 mmol) 94% 6-methoxytryptophan.

2.2.6.1. 4-Methoxytryptophan

Decomposition: 245–250°C.

^{1}H NMR (400 MHz, D_2O): δ=2.88 (dd, 1H, β-H_a), 3.15 (dd, 1H, β-H_b), 3.45 (dd, α-H), 3.84 (s, 3H, CH_3O), 6.53 (d, 1H, H-7), 6.96 (s, 1H, H-2), 7.00 (d, 1H, H-5), 7.02 (dd, 1H, H-6) ppm. $^3J_{\alpha,\beta}$= 7.2, $^2J_{\beta a,\beta b}$=14.7, $^3J_{5,6}$=7.0, $^3J_{6,7}$=7.1 Hz.

^{13}C NMR (100 MHz, D_2O): δ=34.6 (CH_2), 57.9 (CH_3O), 60.4 (CH), 102.4 (C-7), 108.0 (C-2), 113.5 (C-3), 120.2 (C-3a), 125.2 (C-6), 125.9 (C-5), 135.3 (C-7a), 156.3 (C-4), 185.6 (COOH) ppm.

MS (70 eV, EI): m/z (%)=234 (6, M^+), 187 (10), 161 (13), 160 (100), 145 (10), 144 (6), 132 (5), 117 (9), 94 (2), 89 (3), 77 (3), 63 (4).

2.2.6.2. 6-Methoxytryptophan

Decomposition: 240–250°C.

^{1}H NMR (400 MHz, D_2O): δ=2.85 (dd, 1H, β-H_a), 3.03 (dd, 1H, β-H_b), 3.43 (dd, 1H, α-H), 3.75 (s, 3H, CH_3O), (dd, 1H, H-5), 6.96 (d, 1H, H-7), 7.02 (s, 1H, H-2), 7.51 (d, 1H, H-4) ppm. $^3J_{\alpha,\beta}$=5.1, $^2J_{\beta a,\beta b}$=14.2, $^3J_{4,5}$=8.8, $^4J_{5,7}$=2.4 Hz.

^{13}C NMR (100 MHz, D_2O): δ=31.4 (CH_2), 56.2 (CH_3O), 56.9 (CH), 96.0 (C-7), 108.7 (C-5), 111.2 (C-3), 120.0 (C-4), 123.1 (C-3a), 124.2 (C-2), (C-7a), 156.3 (C-6), 183.2 (COOH) ppm.

MS (70 eV, EI): m/z (%)=234 (8, M^+), 188 (4), 161 (14), 160 (100), 145 (10), 132 (5), 132 (5), 117 (9), 95 (2), 89 (3), 77 (2), 63 (2).

2.2.7. 4- and 6-Hydroxytryptophan (5a,b)

The reaction was carried out in a 4 ml screw cap amber vial under nitrogen. 100 mg (427 µmol) of the respective methoxytryptophan were suspended in 2 ml sulfolane. Then 72 µl (512 µmol) triethylamine and 700 µl (5.2 mmol) trimethylsilyl iodide were added and the red solution was stirred and heated for 1 h at 50°C. The intermediate trimethylsilyl ethers formed during the reaction were cleaved and excess trimethylsilyl iodide was decomposed by addition of 2 ml methanol. After adjusting the pH to 4.0 with 1 *M* ammonia the solution was applied to a column (10×50 mm), filled with cation-exchange resin Dowex-50W (H^+ form). The column was washed first with 6 ml methanol–water (1:1, v/v), then with 10 ml water and eluted with 0.5 *M* ammonia at a flow-rate of 0.5 ml/min. The product fraction (pH 8.0) was collected and applied to a column (10×50 mm), filled with anion-exchange resin Dowex-1 (CH_3COO^- form). The column was washed with 15 ml water and eluted with 0.5 *M* acetic acid at a flow-rate of 0.5 ml/min. After evaporation of the solvent, 30 mg (136 µmol) 34% 4-OH-TRP and 60 mg (274 µmol) 43% 6-OH-TRP were obtained as grey crystals.

2.2.7.1. 4-Hydroxytryptophan

Decomposition: >300°C.

^{1}H NMR (400 MHz, D_2O): δ=3.25 (dd, 1H, β-H_a), 3.64 (dd, 1H, β-H_b), 4.13 (dd, 1H, α-H), 6.55 (dd, 1H, H-6), 7.07 (d, 1H, H-5), 7.08 (d, 1H, H-7), 7.14 (s, 1H, H-2) ppm. $^3J_{\alpha,\beta}$=8.1, $^2J_{\beta a,\beta b}$=14.8, $^3J_{5,6}$=7.2, $^3J_{6,7}$=7.2 Hz.

^{13}C NMR (100 MHz, D_2O): δ=35.3 (CH_2), 61.4 (CH), 102.6 (C-7), 108.3 (C-2), 114.5 (C-3), 120.2 (C-3a), 125.2 (C-6), 125.9 (C-5), 135.3 (C-7a), 156.3 (C-4), 185.6 (COOH) ppm.

MS (70 eV, EI): m/z (%)=220 (6, M^+), 173 (7), 147 (11), 146 (100), 145 (14), 133 (43), 132 (5), 117 (3), 104 (5), 91 (7), 78 (10), 76 (6), 65 (9).

2.2.7.2. 6-Hydroxytryptophan

Decomposition: >310°C.

^{1}H NMR (400 MHz, D_2O): δ=3.23 (dd, 1H, β-H_a), 3.42 (dd, 1H, β-H_b), 4.03 (dd, 1H, α-H), 6.77 (dd, 1H, H-5), 6.96 (d, 1H, H-7), 7.16 (s, 1H, H-2), 7.56 (d, 1H, H-4) ppm. $^3J_{\alpha,\beta}$=8.1, $^2J_{\beta a,\beta b}$=15.8, $^3J_{4,5}$=8.1, $^3J_{5,7}$=2.0 Hz.

^{13}C NMR (100 MHz, D_2O): δ=30.8 (CH_2), 58.2 (CH), 96.0 (C-7), 108.7 (C-5), 111.2 (C-3), 120.0 (C-4), 123.1 (C-3a), 124.2 (C-2), (C-7a), 150.6 (C-6), 183.2 (COOH) ppm.

MS (70 eV, EI): m/z (%)=220 (8, M^+), 174 (6), 147 (18), 146 (100), 145 (10), 133 (28), 132 (5), 117 (9), 104 (5), 91 (7), 78 (10), 65 (9).

2.2.8. 7-Hydroxytryptophan

A suspension of 220 mg (710 μmol) 7-benzxyloxy-tryptophan, 140 mg palladium on charcoal (10%), 5 ml ethanol and 5 ml water was hydrogenated at room temperature and atmospheric pressure for 2 h. After that, the catalyst was filtered off and the clear light-yellow filtrate was diluted with water and freeze dried, yielding 133 mg (610 μmol) 86% of 7-OH-TRP.

^{1}H NMR (400 MHz, D_2O): δ=3.25 (dd, 1H, β-H_a), 3.44 (dd, 1H, β-H_b), 4.03 (dd, 1H, α-H), 7.63 (d, 1H, H-5), 7.04 (dd, 1H, H-5), 7.16 (s, 1H, H-2), 7.56 (d, 1H, H-4) ppm. $^3J_{\alpha,\beta}$=8.1, $^2J_{\beta a,\beta b}$=15.2, $^3J_{4,5}$=8.1, $^3J_{5,7}$=2.0 Hz.

NMR analysis ^{1}H and ^{13}C NMR spectra (solvents [D_1]-$CDCl_3$ and [D_2]-D_2O, external standard tetramethylsilane) were recorderd with a 400 AMX (400 MHz) instrument (Bruker, Rheinstetten, Germany).

Mass spectra were recorded with a Varian 311 (70 eV) (Darmstadt, Germany).

2.3. Irradiation

Irradiation with the doses of 1, 3 and 5 kGy (dose rate: 0.7 kGy/h) was performed by a ^{137}Cs source at Beiersdorf AG (Hamburg, Germany). Irradiation with the doses of 10 and 20 kGy (dose rate: 0.5 kGy/h) was carried out using an industrial ^{60}Co source at Beiersdorf AG.

2.4. Sample treatment

Solutions of TRP (400 mg/l and 200 mg/l) in bidistilled water were filled in 20 ml polyethylene vials (Zinsser, Frankfurt, Germany) for irradiation. Soon after irradiation the solutions were deep frozen at −20°C. After thawing, these solutions were filtered (0.2 μm) and directly used for HPLC analysis.

All investigations were carried out at least in triplicate. For control, non-irradiated blind samples were treated in the same way.

2.5. HPLC analysis

A Merck L-6200 pump was used for HPLC analysis. Detector signals were analyzed by Bruker Chromstar software (Bremen, Germany).

The separation of the radiation products was performed on a Nucleosil 120-3 RP 18 column (250×4 mm) using a ternary gradient of TFA (0.1% in bidistilled water), MeOH and MeCN (HPLC conditions see Table 1). The eluting substances were monitored with two detectors in series. The first was a Merck Hitachi L 4000 UV detector set at 260 nm, the second was a Merck Hitachi F 1080 fluorescence detector set at 270 nm for excitation and 315 nm for emission.

UV spectra (400–200 nm) of the products were obtained by a Waters 994 programmable photodiode array detector. Fluorescence spectra (λ_{em}=280–400 nm, λ_{ex}=200–320 nm) were performed with the fluorescence detector mentioned above.

3. Results and discussion

3.1. Synthesis of 4-, 6- and 7-OH-TRP

The desired compounds were obtained in sufficient amounts and in purities which allow their use as reference substances for analytical studies. 7-OH-TRP was obtained by hydrogenation of 7-benzyloxy-tryptophan in a one step reaction with a purity of >98% (HPLC–UV 260 nm) [10].

Table 1
HPLC conditions for the separation of TRP-irradiation products (Nucleosil 120-3 RP18 column, 250×4 mm)

		0.1% TFA (%)	Methanol (%)	Acetonitrile (%)
Gradient	0 min	95	5	0
	10 min	86	14	0
	25 min	66	14	20
	26 min	46	14	40
	30 min	46	14	40
	31 min	95	5	0
	45 min	95	5	0
Flow		1 ml/min		
Temperature		35°C		
Injection volume		20 μl		

Ek and Witkop [10] were the first to report a synthesis of 5- and 7-hydroxytryptophan. Using methylnitrophenoles as starting material, they obtained the respective benzyloxyindoles in a time intensive five step procedure. The attempt, to synthesize 4- and 6-OH-TRP according to their method failed in the first step. In contrast, the synthesis of 4- and 6-methoxyindole according to Sugasawa et al. [9], who performed a two step synthesis of these products from 3-methoxyaniline was successful. The formation of the amino acid side chain to the respective methoxytryptophanes was achieved via the gramines [10] (scheme of synthesis see Fig. 1). The last step within this synthesis was the cleavage of the methoxy protective group by treatment with trimethylsilyl iodide [11]. The modest yields are due to the formation of HI in situ and the oxidative conditions which cause a partial destruction of the labile indole ring.

An efficient synthesis strategy was found to obtain 4- and 6- OH-TRP in moderate yields and high purities (6-OH-TRP >98%, 4-OH-TRP >90%, HPLC–UV 260 nm).

3.2. Irradiation of TRP

It was possible to separate the supposed radiation products of TRP in one single run and to determine them beside TRP. Fig. 2 shows a typical chromatogram of a γ-radiated TRP-solution. The identification of the radiation products was done by comparison of the retention times with those of reference substances and by standard addition. To confirm these results, the UV spectra of the radiation products, obtained by PDA-detection, were compared with those of reference substances. In addition, fractions of fluorescent radiation products were collected during HPLC separation, and the FL-spectra ($\lambda_{ex}/\lambda_{em}$, 270/315 nm) of these fractions were compared with those of reference substances.

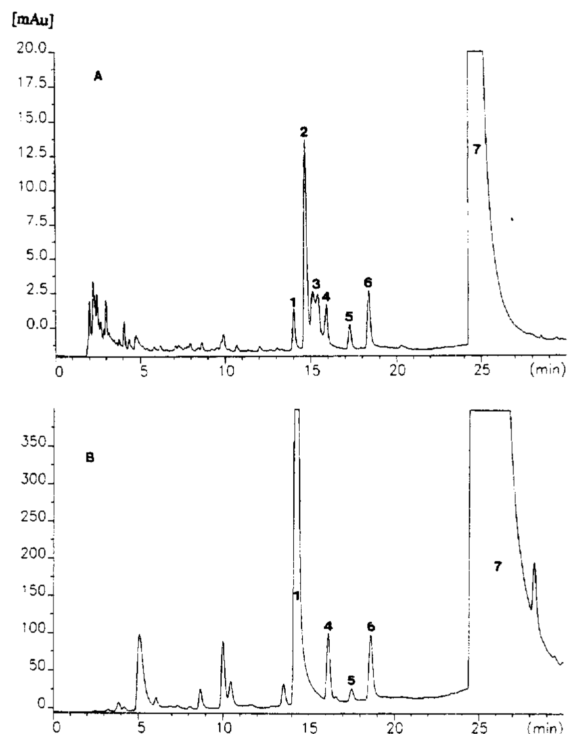


Fig. 2. Chromatogram of an irradiated TRP solution (400 mg TRP/l, 10 kGy). (A) UV-detection (260 nm), (B) fluorescence detection (λ_{ex}=270 nm, λ_{em}=315 nm). (1) 5-OH-TRP, (2) NFK, (3) OIA, (4) 6-OH-TRP, (5) 4-OH-TRP, (6) 7-OH-TRP, (7) TRP.

In addition to the six main primary radiation products NFK, OIA and the four OH-TRP isomers (4-, 5-, 6- and 7-OH-TRP), traces of KYN, and DIOIA were detected (structures see Fig. 3). The OH-TRP isomers could be detected as well by UV detection as by fluorescence detection. The other radiation products show no fluorescence under these conditions (Fig. 3).

These results correspond well with the results of other authors. In γ-radiated TRP solutions Troeder and Steinhart [2] identified NFK, KYN, and 5-OH-TRP by HPLC separation and UV detection. They also detected OIA and further OH-TRP isomers by GC–MS. The OH-TRP isomers showed the same mass and the same fragmentation as 5-OH-TRP but an identification of the single substances was not possible. Singh et al. [5] identified NFK, KYN, and 5-OH-TRP by HPLC and UV detection. Additionally they detected further peaks, which they could not identify but assumed them to be OH-TRP adducts. Maskos et al. [1] determined OIA, NFK, 4-, 5-, 6- and 7-OH-TRP in γ-radiated TRP solutions with HPLC–UV and electrochemical detection. Because of the lack of 4-, 6- and 7-OH-TRP as reference substances they compared the UV-spectra of the corresponding OH-indols with those of the OH-TRP for identification. In contrast to these results a sufficient identification of the OH-TRP in γ-radiated solutions of TRP was possible using the synthesized substances.

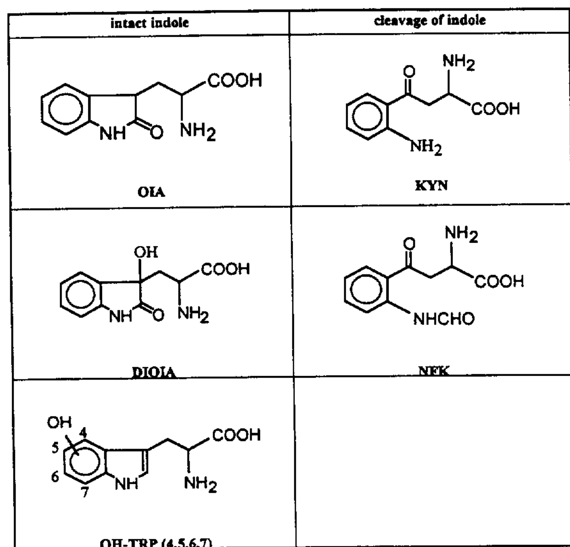


Fig. 3. Radiation products of TRP.

Additionally the γ-radiated solutions showed yellow to brown coloration in relation to the applied radiation dose. This effect probably depends on polymerization as the result of radical–radical reactions and has also been observed by various other authors [1,2,4].

3.3. Quantification of the main radiation products

The quantification of the main radiation products was performed by the method of external standard. Calibration curves (UV 260 nm) were recorded for all determined substances. In addition, calibration curves with the FL detector ($\lambda_{ex}/\lambda_{em}$, 270/315 nm) were recorded for the OH-TRP isomers. The detector response was linear for all examined substances. The detection limits (signal-to-noise ratio of 3:1) of the examined radiation products are described in Table 2.

The relation between the radiation products and the applied radiation dose on a TRP solution (400 mg/l) is illustrated in Fig. 4. Up to the radiation dose of 5 kGy all examined substances show an increase with increasing radiation doses. Between the radiation dose of 10 and 20 kGy a difference in the behavior of the substances can be observed. While the amounts of OH-TRP isomers and OIA are still increasing or remain on the same level, the amount of NFK decreases. The amounts of the single substances are also different. The OH-TRP isomers were formed from 0.1% up to 2% in relation to the initial TRP content, while the values of OIA were between

Table 2
Detection limits of the determined radiation products

Substance	UV (260 nm) (μg/l)	Fluorescence ($\lambda_{ex}/\lambda_{em}$ 270/315 nm) (μg/l)
OIA	60	
DIOIA	70	
KYN	20	
NFK	30	
4-OH-TRP	120	250
5-OH-TRP	40	7
6-OH-TRP	95	60
7-OH-TRP	130	180

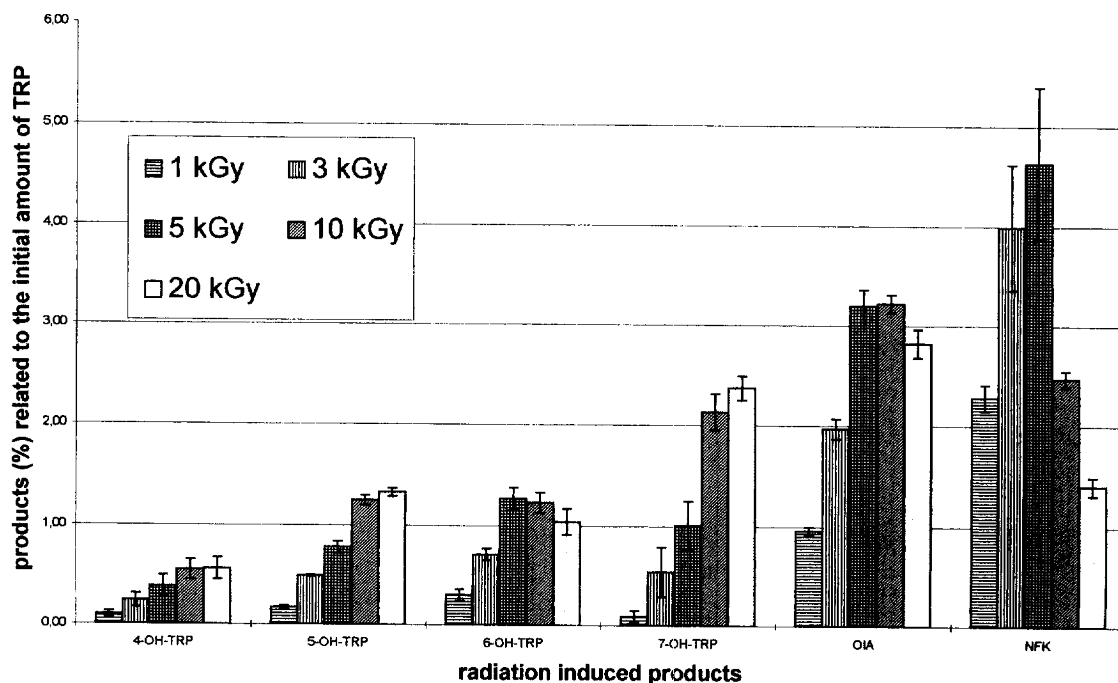


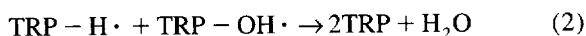
Fig. 4. Formation of the radiation induced products of TRP (400 mg/l) related to the initial amount of TRP (%). Values are the mean of five determinations. Standard deviation is given by the T-bars.

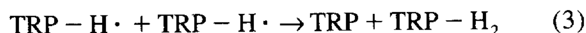
1% and 3,2% and those of NFK between 1.4% and 4,6% of the original TRP content. None (<0.03% in relation to the origin TRP content) of the examined radiation products was detected in the blind samples. Similar results were observed in solutions of lower TRP concentration (200 mg TRP/l). Up to 3 kGy the sum of the examined radiation products was relatively greater than that of 400 mg TRP/l, whereas it was smaller between 5 and 20 kGy (see Table 3). A remarkable difference was the decrease of radiation products between 10 and 20 kGy in the solutions of lower concentration.

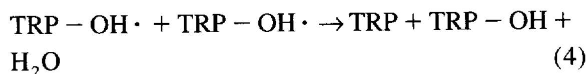
In solutions of lower TRP concentration and radiation doses of more than 3 kGy more substances are formed, which are not detectable with the applied methods. A comparison between the relation of the radiation induced loss of TRP and the sum of the determined radiation products (Table 3) shows that the sum of the detected products amounts to only a small part of the TRP destruction. Further, the destruction of TRP is relatively smaller in the solution with 400 mg TRP/l than that in the less concentrated solution. Probably more recombination reactions (see Eqs. (2)–(4)) between TRP radicals are possible in solutions with a higher TRP concentration [12].

Table 3
Comparison of the loss of TRP and the sum of the determined radiation products

TRP (mg/l)		Radiation dose (kGy)				
		1	3	5	10	20
400	TRP loss (%)	3.9	21.7	32.3	57.6	80.6
400	Sum of radiation products (%)	3.2	7.9	11.3	10.9	9.5
200	TRP loss (%)	11.6	58.3	64.5	85.1	98.1
200	Sum of radiation products (%)	5.9	9.3	8.4	6.8	2.1


$$TRP - H\cdot + TRP - OH\cdot \rightarrow 2TRP + H_2O \quad (2)$$


$$TRP - H\cdot + TRP - H\cdot \rightarrow TRP + TRP - H_2 \quad (3)$$


$$TRP - OH\cdot + TRP - OH\cdot \rightarrow TRP + TRP - OH + H_2O \quad (4)$$

3.4. Radiation products of TRP as possible markers for irradiated food

NFK and OIA should be suitable markers for irradiated food because of the high amounts in which they were formed during radiation. But as NFK is a metabolite of TRP and is therefore formed during normal biological processes and OIA is probably formed during all oxidation processes of TRP [13], e.g., food processing, these products are not appropriate for marker substances.

Although the OH-TRP isomers are formed in smaller amounts, they could be detected very selectively and sensitively because of their fluorescence. 5-OH-TRP is a physiological metabolite of TRP and therefore not suitable as marker substance for irradiated food. 4-, 6- and 7-OH-TRP are not known to be formed during the metabolism of TRP or during food processing. Therefore they could possibly used as marker substances for irradiated food with a high protein content.

Acknowledgments

This work was supported by the "Deutsche Forschungsgemeinschaft". We thank Dr. Bogdahn from Beiersdorf AG for assistance with the γ-radiation.

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